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Vanadate inhibits urinary acidification by the turtle bladder

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Vanadate inhibits urinary acidification by the turtle bladder. We studied the effect of vanadate on urinary acidification by the turtle bladder in vitro. Vanadate added to the serosal, but not to the mucosal, solution caused a dose dependent inhibition of hydrogen ion secretion in short circuited hemibladders in the presence and in the absence of ouabain. The failure of mucosal addition of vanadate to inhibit hydrogen ion secretion was due to the fact that vanadate tissue uptake from the serosal solution was 30 times greater than that from the mucosal solution, suggesting that vanadate must enter the cell to inhibit hydrogen ion secretion. The effect of vanadate on hydrogen ion secretion could not be explained by alterations in calcium uptake or efflux. The effect of vanadate on hydrogen ion secretion under aerobic conditions could not be explained solely by inhibition of mitochondrial function because the relative magnitude of the inhibitory effect of this compound was the same under aerobic and anaerobic conditions. Vanadate caused a significant inhibition of the proton motive force (PMF) without altering backleak of hydrogen ion or bicarbonate secretion. Under anaerobic conditions vanadate inhibited hydrogen ion secretion and caused a significant increase in lactate production without the expected increase in ATP/ADP ratio. ATP levels under anaerobic conditions were not significantly different between control and vanadate treated hemibladders, indicating that limited ATP availability can not explain the inhibitory effect of vanadate on hydrogen ion secretion. These findings indicate that vanadate has a number of effects that are capable of explaining the inhibition of urinary acidification. Among those effects, the inhibition of hydrogen ion secretion and the PMF by vanadate may be due to direct inhibition of the hydrogen ion pump, interference with ATP utilization by the pump, or other metabolic effects.

Le vanadate inhibe l'acidification urinaire par la vessie de tortue. Nous avons étudié in vitro l'effet du vanadate sur l'acidification urinaire par la vessie de tortue. La présence de vanadate dans le bain séreux, mais non dans le bain muqueux, détermine une inhibition, dépendante de la dose, de la sécrétion d'ions d'hydrogène dans les hémi-vessies en court-circuit, en présence et en l'absence d'ouabaïne. L'absence d'effet de l'addition de vanadate à la face muqueuse est due au fait que la captation tissulaire de vanadate est 30 fois plus grande à partir du bain séreux que du bain muqueux, ce qui suggère que le vanadate doit pénétrer dans la cellule pour inhiber la sécrétion d'ion hydrogène. L'effet du vanadate sur la sécrétion d'ions d'hydrogène ne peut être expliqué par les modifications de la captation ou de l'efflux de calcium. L'effet du vanadate sur la sécrétion d'ions d'hydrogène dans des conditions aérobies ne peut être expliqué seulement pas l'inhibition de la fonction mitochondriale puisque l'importance relative de l'effet inhibiteur est la même dans les conditions aérobies et anaérobies. Le vanadate détermine une inhibition significative de la force motrice des protons (PMF) sans modification de la rétrodiffusion d'ions d'hydrogène ou de la sécrétion de bicarbonate. En condition anaérobie, le vanadate inhibe la sécrétion d'ions d'hydrogène et détermine une augmentation significative de la production de lactate sans l'augmentation attendue du rapport ATP/ADP. Les concentrations d'ATP en condition anaérobie ne sont pas significativement différentes entre les hémi-vessies contrôles et traitées par le vanadate, ce qui indique que la disponibilité d'ATP ne peut expliquer l'effet inhibiteur du vanadate sur la sécrétion d'ions d'hydrogène. Ces constatations indiquent que le vanadate a de nombreux effets qui sont capables de participer à l'inhibition de l'acidification urinaire. Parmi ces effets l'inhibition de la sécrétion d'ions d'hydrogène et de la PMF par le vanadate peut être due à une inhibition directe de la pompe ions d'hydrogène, à une interférence avec l'utilisation d'ATP par la pompe ou d'autres effets métaboliques.

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In recent years the effect of vanadate on several transport processes has been studied extensively [1–10]. Vanadate inhibits the Na-K-ATPase by blocking its conformational change that follows hydrolysis of the phosphoenzyme [4, 6]. Recent studies have also demonstrated that vanadate is capable of inhibiting proton ATPases of plasma membrane of several fungi [11–13]. The turtle urinary bladder is capable of acidification in vitro. In this membrane acidification is thought to be mediated by a proton translocating ATPase [14–16]. These observations therefore suggested to us that vanadate may be used to probe the mechanism of urinary acidification in the turtle bladder.

Methods

The methods used in the present experiments have been previously described [17–20]. In brief, urinary bladders of freshwater turtles *(Pseudemys scripta)* were removed, washed in Ringer's solution, and divided into halves; each hemibladder was mounted in a plastic chamber (Lucite). The exposed area of the bladder was 8 cm^2, and the results are expressed for this area. The two sides of the bladder were bathed in Ringer's solution containing the following composition, in millimoles per liter: sodium chloride, 114.4; potassium chloride, 3.5; dibasic sodium phosphate, 2.0; dextrose, 5; magnesium chloride, 0.5; and calcium chloride, 1.8 (pH, 7.4; osmolality, 249 mOsm/kg H_2O). In most experiments, the mucosa was bubbled with compressed air, and the serosa was bubbled with a gas mixture of 99% air and 1% carbon dioxide. The spontaneous potential difference (PD) was measured by bridges of 3 M potassium chloride and agar and half cells of calomel connected to a

voltmeter (Keithley model 600B, Keithley Instruments, Inc. Cleveland, Ohio). An automatic voltage clamp was used to supply enough current via the potassium chloride and agar bridges; silver chloride electrodes were used to nullify the spontaneous PD. The short circuit current (SCC) was measured by a Simpson micrometer and recorded continuously with a Fisher recorder. Bladders that failed to maintain a spontaneous PD greater than 15 mV during the first hour were discarded. The rate of mucosal acidification was measured as the reverse short circuit current (RSCC) after sodium transport was abolished by ouabain (5×10^{-4} M) added to the serosal solution [21]. In some experiments hydrogen ion secretion was measured with the pH stat method as previously described; unless otherwise indicated, the pH of the mucosal and serosal solution was maintained at 7.4. In the experiments reported here, the RSCC was recorded continuously except for brief intervals when the PD was measured. After baseline rate of hydrogen ion secretion was stable, sodium orthovanadate, diluted in turtle Ringer's solution, was added to the serosal or to the mucosal solution, and an equal amount of vehicle was added to control hemibladders. In some experiments, 5% carbon dioxide and 95% air were added to the serosal solution to attempt to reverse the inhibitory effect of vanadate on hydrogen ion secretion. Experiments were also performed in which the mucosa was bubbled with 100% nitrogen, and the serosal solution was bubbled with 95% nitrogen and 5% carbon dioxide for the whole duration of the experiments.

In some experiments, the pH gradient necessary to nullify the rate of hydrogen ion secretion (designated as the apparent proton motive force) was measured before and after 1 hour of addition of the vanadate or vehicle to the serosal side [18, 22]. In these experiments, the pH of the mucosa was lowered in a stepwise fashion from 7.4 to 6.4, 5.4, and then to a level at which hydrogen ion secretion was nullified. At each of these levels hydrogen ion secretion was measured. The apparent PMF (expressed in pH units) was calculated as the difference between the serosal pH (7.4), and the mucosal pH at which hydrogen ion secretion was nullified. The active conductance of protons was calculated as the slope of hydrogen ion secretion against the pH gradient by linear regression analysis. In all experiments, the relationship between hydrogen ion secretion, and pH gradient was linear with a correlation coefficient greater than 0.9.

In the experiments where the rate of alkali entry into the mucosal solution was measured, the pH stat technique was used, with 0.01 N hydrochloric acid as the titrant [17]. In these experiments, two Ringer solutions with identical chloride concentrations and osmolalities were used. The first Ringer's solution was the control solution and contained the following, in millimoles per liter: sodium sulfate, 13.3; sodium chloride, 94.4; potassium chloride, 3.5; magnesium chloride, 0.5; dibasic sodium phosphate, 2.0; calcium chloride, 1.8; and dextrose, 5 (pH, 7.4; osmolality, 236 mOsm/kg H_2O). The second bicarbonate Ringer's solution contained sodium bicarbonate, 20; sodium chloride, 94.4; potassium chloride, 3.5; magnesium chloride, 0.5; dibasic sodium phosphate, 2.0; calcium chloride, 1.8; and dextrose, 5 (pH, 8; osmolality, 236 mOsm/kg H_2O). In these experiments, the bladders were mounted and bathed with the control Ringer's solution. Ouabain, 5×10^{-4} M, was added to the serosal solution. The pH of the serosa was maintained at 8, whereas the pH of the mucosa was lowered below 5 with hydrochloric acid. The pH of the mucosa at which net hydrogen ion secretion became zero was determined. After the end-point was achieved, the serosal solution was replaced with bicarbonate Ringer's solution, and the rate of 0.01 N hydrochloric acid required to maintain the pH at the end-point level was measured. Only rates of bicarbonate secretion that remained stable for at least 30 min were analyzed.

In the experiments designed to assess the rate of hydrogen loss from the mucosa, the bladders were bathed with regular turtle Ringer's solution, and the mucosal pH at which net hydrogen ion secretion became zero was determined [17]. Hemibladders with end-points higher than 5.1 were not included in the study. The rate of hydrogen loss was determined by titrating the mucosa with 0.01 N hydrochloric acid to keep the pH of the mucosa constant at the point of zero net hydrogen ion secretion [17].

Vanadate fluxes. In these experiments, paired hemibladders were mounted either in the presence or absence of ouabain; after the SCC (sodium transport) or the RSCC (hydrogen ion transport) was stable, sodium orthovanadate 10^{-4} M was added to the serosal solution of one hemibladder and to the mucosal solution of the other hemibladder. Vanadyl chloride $^{48}VOCl_2$ was obtained from Amersham, and it was oxidized to vanadate as described by Day et al [10]. One microcurrie of gamma-emitting sodium vanadate ($^{48}VO_4$; specific activity, 100,000 to 500,000 cpm/mole) was added to the mucosal bath of one hemibladder and to the serosal bath of the other hemibladder. Aliquots of the baths were taken immediately after addition of $^{48}VO_4$. A the end of 60 min, the radioactivity of all the baths was determined, and the serosal to mucosal, mucosal to serosal, and the net fluxes were calculated. Then the hemibladders were removed carefully from the chambers with a scalpel, dipped in 10 ml of turtle Ringer's containing 10 mM norepinephrine [10], blotted, weighed, and counted. The amount of vanadate taken up by the tissue was calculated, and the results are expressed as picomoles/mg of tissue wt per 60 min.

Uptake of ^{48}vanadate by isolated turtle bladder epithelial cells. The mucosal layer of the bladders was stripped [19] and incubated for 90 min in oxygenated turtle Ringer's containing 5 mg/ml of collagenase at 28° C. The cells were spun at $\times 5000g$ for 10 min, resuspended in 3 ml of buffer and carefully mixed to attain an even suspension. They were gently washed and recentrifuged two additional times. An aliquot of the final cell suspension was taken for determination of cell protein, and another containing 2 to 4 mg protein was incubated at 20° C with 2.5 ml of turtle Ringer's containing either 10^{-4} or 10^{-5} M cold orthovanadate and 1 μCi of $^{48}VO_4$ (specific activity, 100,000 to 500,000 cpm/mole). The pH of the turtle Ringer was 7.4, and the cell suspension was continuously bubbled with compressed air for 5, 10, or 20 min. At the times specified, the reaction was stopped by the addition of 6 ml of turtle Ringer's containing 10 mM norepinephrine and centrifuged 5 min at $\times 5000g$ at 4° C. The supernatant was decanted, and the cell pellet washed two additional times. The final pellet was resuspended in 1 ml of buffer and counted in a gamma counter. A blank was run with each series of experiments in which the labeled medium without cells was handled in a manner identical to the experimental protocol. The cpm of the final centrifugation was not different from background (40.0 ± 3.5). The results

are expressed as picomoles of VO_4 uptake per milligram of protein, over the time period examined.

Assay of ATP in turtle bladder epithelial cells. At the end of each experiment (in which paired hemibladders were studied), the hemibladders were rapidly removed from the chambers, washed with cold Ringer's solution, and pinned (mucosal side up) on frozen paraffin disks. This required less than 30 sec. The tissues were immersed immediately in liquid nitrogen, and the mucosal surface was scraped gently with a scalpel that was dipped into liquid nitrogen. The scraped material was placed into 3 ml of frozen 6% perchloric acid and allowed to thaw for 30 min. The tissue suspension was centrifuged at $\times 39{,}100g$ (Sorvall RC2-B, Newtown, Connecticut) at 4° C for 10 min. The supernatant was made alkaline to a phenolphthalein end-point (pH approximately 8.2 to 10.0) with a solution containing 7.5 M potassium hydroxide and 0.1 M dibasic potassium phosphate, spun at $30g$ for 2 min, and maintained at 4° C. The supernatant was used to measure ATP.

Extraction and activation of luciferin-luciferase. ATP was measured as reported by Kimmich, Randles, and Brand [24]. Desiccated firefly lanterns were obtained from Sigma (St. Louis, Missouri) and stored at −20° C. Fifty milligrams of lanterns were homogenized in 5.0 ml of buffered medium in a glass homogenizer fitted with a Teflon™ pestle. The homogenizing medium contained 50 mM sodium arsenate, 20 mM magnesium sulfate, and 20 mM sodium glycylglycine (pH, 7.4). The homogenate was centrifuged at $\times 18{,}000g$ for 10 min at 4° C, and the supernatant was further activated with 80 mg of solid calcium phosphate. The resulting slurry was allowed to stand at room temperature for 10 min with occasional mixing. The suspension was centrifuged at $\times 400g$ for 2 min at 4° C and the calcium phosphate step repeated. Thereafter, the mixture was spun at $\times 18{,}000g$, 4° C for 10 min. The fresh supernatant was used for the ATP assay.

ATP assay. Background and ATP-induced luminescense of the firefly lantern extract was monitored in a Beckman LS 230 liquid scintillation counter (Beckman, Fullerton, California) operated at room temperature. Routine assays were performed in standard scintillation vials containing 0.9 ml of assay medium (5 mM sodium arsenate, 4 mM magnesium sulfate, 20 mM glycylglycine; pH, 8.0) and 50 μl of unknown sample or ATP standard. Fifty microliters of activated lantern extract were added to start the light producing reaction. Exactly 20 sec after addition of the extract, the well-mixed incubate was counted for 30 sec. ATP standards were prepared from ATP obtained from LKB instruments, Inc. (Rockville, Maryland). Freshly prepared or frozen ATP standards were enzymatically pretreated to convert ADP and AMP (resulting from spontaneous dephosphorylation of ATP) to ATP. ADP was enzymatically converted to ATP by treatment with phosphoenolpyruvate and pyruvate kinase (this technique was also used to measure ADP levels; see Results), and AMP was converted to ATP by inclusion of myokinase in the reaction mixture [24]. These conversion steps resulted in an increase in ATP dependent luminescense of 3 to 22%. ATP concentration was expressed in nanomoles per milligram of protein. All enzymes and other reagents were obtained from Sigma (St. Louis, Missouri).

Lactate production and calcium 45 transport. The serosal solution of ouabain treated hemibladders was bubbled with 95% nitrogen, 5% carbon dioxide (see above) and the mucosal solution with ouabain treated hemibladders was bubbled with 95% nitrogen, 5% carbon dioxide (see above) and the mucosal solution of 100% nitrogen. After the hydrogen ion current achieved a stable value, 10^{-4} M sodium orthovanadate was added to the serosal solution of the experimental hemibladders, and the vehicle was added to the control. The hydrogen ion current was recorded for 90 min after addition of vanadate. Before and after addition of vanadate, 1 ml of serosal solution was sampled and replaced by 1 ml of fresh turtle Ringer's at 60-min intervals for determination of lactate [25].

Lactate was measured in timed 1-ml aliquots from the serosal bath of the anaerobically mounted turtle bladders using a commercially available spectrophotometric assay (Sigma, St. Louis, Missouri). The samples were taken to dryness under a stream of air and taken up in 0.3 ml of distilled H_2O. Lactic acid standards were equally treated. All samples were determined in triplicate, and it was assured that the reaction had reached completion. Lactate production was expressed in micromoles per hour.

The methods used to measure calcium uptake and efflux were identical to those previously described by us [19].

Data are presented as mean ± SEM. Student's *t* test for paired or unpaired data was used whenever appropriate. The data for hydrogen ion secretion was normalized by expressing the results as percentage of baseline values.

Results

Effect of vanadate on hydrogen ion secretion. Figure 1 (left panel) shows the effect of vanadate on hydrogen ion secretion (measured as the RSCC), expressed as percentage of baseline values. In the presence of 99% air and 1% carbon dioxide in the serosal solution, 10^{-4} M vanadate added to the serosal solution caused a rapid decline in hydrogen ion secretion; after 10 min of observation hydrogen ion secretion was 72.9% of baseline values (Fig. 1, left panel). Hydrogen ion secretion continued to decline achieving a mean inhibition of 59.0 ± 7.91 of baseline values at the end of 1 hour. In control hemibladders, hydrogen ion secretion was unchanged. Baseline hydrogen ion secretion was not different between control and experimental hemibladders (46.6 ± 8.0 vs. 50.7 ± 14.1 μA, NS). Addition of vanadate to the mucosal solution, in a concentration of 10^{-4} M, failed to alter hydrogen ion secretion. In another group of six hemibladders, hydrogen ion secretion was measured by pH stat technique in the absence of ouabain addition. Hydrogen ion secretion was 44.8 ± 3.9 and 28.1 ± 3.3 μA, $P < 0.001$, before and after addition of vanadate, 10^{-4} M, to the serosal solution.

The effect of vanadate was not reversible with washing of the serosal solution: in six experiments, hydrogen ion secretion was 64.5 ± 5.1% before and 68.7 ± 6.8% 60 min after removal of the bath containing 10^{-5} M vanadate. Addition of 5% carbon dioxide to the serosal solution also failed to reverse the inhibitory effect of vanadate on hydrogen ion secretion. In control hemibladders, 5% carbon dioxide increased hydrogen ion secretion from 100.6 ± 4.2 to 137 ± 7%, $P < 0.001$ of baseline values, whereas in 10^{-4} M vanadate-treated hemibladders hydrogen ion secretion remained unchanged (59.0 ± 7.9 vs 55.5 ± 10.6%, $N = 7$, NS). The difference between control and vanadate-treated hemibladders was highly significant, $P < 0.001$.

The right panel of Figure 1 shows the effect of vanadate on hydrogen ion secretion in bladders bubbled with 100% nitrogen

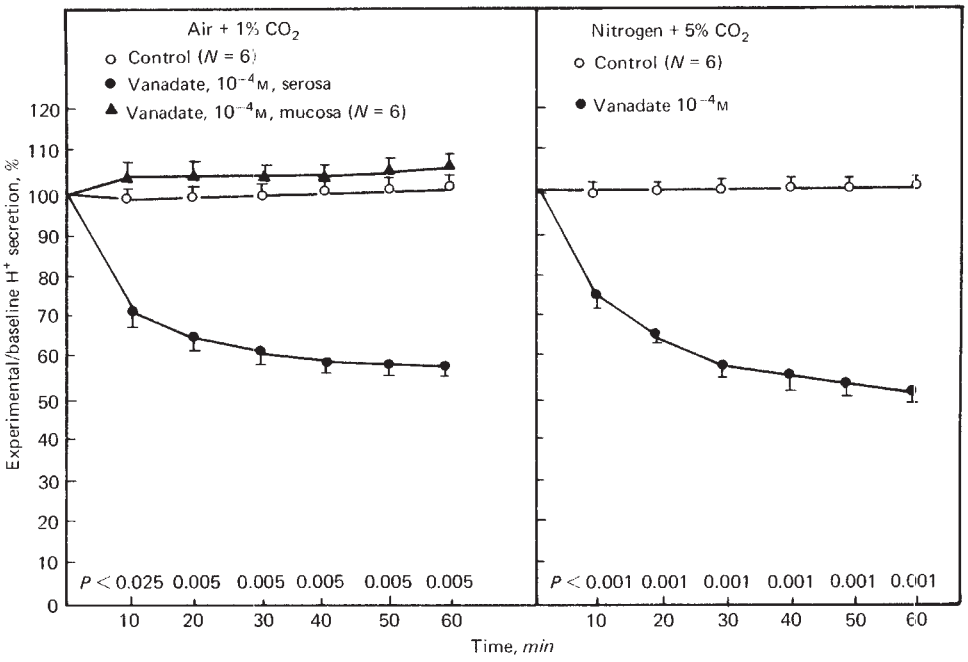

Fig. 1. *Left panel: Hydrogen ion secretion, expressed as percentage of baseline values, in control (N = 6) and in the presence of vanadate added either to the serosal solution (N = 6) or to the mucosal solution (N = 6).* The serosal solution was bubbled with 99% compressed air and 1% carbon dioxide. *Right panel: Hydrogen ion secretion in control and vanadate treated bladders.* Vanadate was added to the serosal solution. The serosal solution was bubbled with 95% nitrogen and 5% carbon dioxide and the mucosal solution with 100% nitrogen.

in the mucosal solution and 95% nitrogen and 5% carbon dioxide in the serosal solution. Baseline hydrogen ion secretion was not different between the two sets of hemibladders (13.1 ± 2.7 and 12.3 ± 1.7 μA, NS). Addition of vanadate, 10^{-4} M, to serosal solution resulted in significant inhibition of hydrogen ion secretion by 5 min; hydrogen ion secretion declined to 52.0 ± 6.1% of baseline values by the end of 1 hour. In control hemibladders, hydrogen ion secretion remained unchanged. The pattern and the relative magnitude of the inhibitory effect of vanadate on hydrogen ion secretion in the presence of nitrogen was very similar to that observed in bladders bubbled with air (Fig. 1, right and left panel). Thus, the inhibitory effect of vanadate on hydrogen ion secretion is not dependent on mitochondrial function.

Figure 2 shows the inhibitory effect of various concentrations of vanadate on hydrogen ion secretion in bladders bubbled with 99% air and 1% carbon dioxide in the serosal solution. The data are expressed as percent of baseline values. Vanadate caused a dose dependent inhibition of hydrogen ion secretion. The inhibition of hydrogen ion secretion was linearly related to the negative log of the molar concentration of vanadate ($y = -37.7 + 22.3x$, $P < 0.001$, $r = 0.961$).

Because vanadate has been suggested to alter microtubule function [26] which itself may inhibit urinary acidification [27], we examined whether pretreatment with colchicine would alter the inhibitory effect of vanadate on hydrogen ion secretion. Colchicine (10^{-4} M) was added to the serosal solution of one hemibladder, and 2 hours later 10^{-4} M vanadate was added to both hemibladders. Addition of colchicine resulted in a decrease in hydrogen ion secretion from 59.9 ± 13.3 to 37.2 ± 11.2 μA, $P < 0.01$, $N = 7$. In control hemibladders, hydrogen ion secretion remained unchanged. Addition of vanadate, 10^{-4} M, to the serosal solution resulted in a comparable net decrease in hydrogen ion secretion in control and colchicine treated hemibladders (control 48.4 ± 10.7, colchicine 40.7 ± 7.9% $N = 7$, NS).

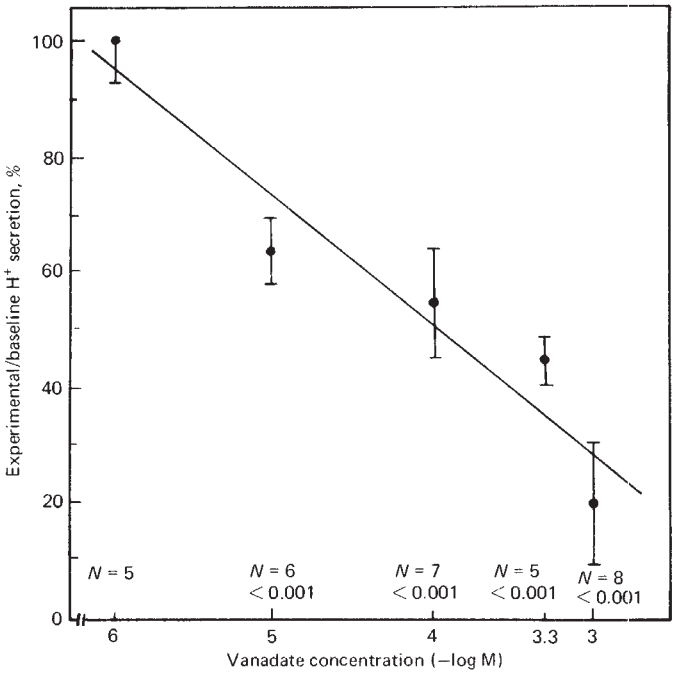

Fig. 2. *Dose response of the effect of vanadate on hydrogen ion secretion.* Hydrogen ion secretion, expressed as percentage at baseline values (ordinate), is plotted against the molar concentration of vanadate (expressed as log of the concentration).

In six additional experiments, we examined whether the effects of vanadate and acetazolamide were additive. Addition of 10^{-4} M vanadate caused a significant decrease in hydrogen ion secretion to 42.7 ± 6.4% of baseline value. Addition of acetazolamide, 5×10^{-4} M, resulted in further decrease in hydrogen ion secretion to 7.2 ± 3.4%, $P < 0.005$.

Table 1. Effect of vanadate, 10^{-4} M, in the serosal solution on the proton motive force (PMF) and active conductance ($N = 8$)[a]

Group	Baseline H^+ secretion μA	Experimental/ baseline H^+ secretion %	PMF, *pH units*			Conductance, $\mu A/pH$ *units*		
			Before	*P*	After	Before	*P*	After
Control	51.2 ± 8.1	107.3 ± 4.0	3.35 ± 0.19	NS	3.52 ± 0.20	14.7 ± 2.2	NS	14.0 ± 1.7
	NS	< 0.001						
Vanadate, 10^{-4}	40.6 ± 7.8	57.2 ± 3.6	2.87 ± 0.30	< 0.02	2.25 ± 0.27	14.4 ± 2.3	< 0.025	9.8 ± 1.4

[a]Values are the means ± SEM.

Table 2. Effect of vanadate (10^{-4} M in the serosal solution) on bicarbonate secretion ($N = 7$)[a]

Group	Bicarbonate secretion, $\mu moles/hr$			Mucosal pH[c] $J_{H=0}$
	Baseline		Experimental[b]	
Control	1.53 ± 0.34	NS	1.44 ± 0.33	4.34 ± 0.08
	NS		NS	NS
Vanadate	1.54 ± 0.22	NS	1.43 ± 0.20	4.28 ± 0.08

[a]Values are the means ± SEM.
[b]Values obtained 60 min after addition of the vehicle or vanadate.
[c]Mucosal pH at which hydrogen ion secretion was nullified.

Effect of vanadate on the proton motive force, passive loss of hydrogen ion from the mucosa and bicarbonate secretion. Table 1 shows the effect of 10^{-4} M vanadate in the serosal solution, on PMF and active conductance. Both the PMF and active conductance of protons were decreased significantly by vanadate. In six additional hemibladders, the effect of 10^{-4} M vanadate on passive hydrogen ion loss from the mucosal solution was examined. Passive hydrogen ion loss from the mucosal solution was undetectable both before and after addition of vanadate to the serosal solution. Table 2 shows that vanadate failed to alter bicarbonate secretion.

Effect of vanadate on lactate production and on ATP levels under anaerobic conditions. To investigate the mechanism of the inhibitory effect of vanadate on hydrogen ion secretion under anaerobic conditions, we evaluated the effect of this compound on both glycolysis, as assessed by lactate production, and ATP levels. Table 3 shows these results. As shown in Figure 1 (right panel), vanadate caused a significant inhibition of hydrogen ion secretion. Vanadate caused a significant increase in lactate production but did not alter total ATP levels. In control hemibladders, all these parameters remained unchanged. In six additional experiments, we measured the ATP and ADP levels in control and vanadate-treated hemibladders under anaerobic conditions. The ATP/ADP ratio was not significantly different between control and vanadate-treated hemibladders (control, 1.09 ± 0.27; vanadate, 1.09 ± 0.22; NS). We also evaluated the effect of vanadate on ATP levels under aerobic conditions in bladders treated with 5×10^{-4} ouabain. At 10^{-3} M, vanadate added to the serosal solution caused a small, though significant, depression of ATP levels (control, 29.7 ± 6.4; 10^{-3} M vanadate, 24.1 ± 4.8 nmoles/mg of protein, $P < 0.05$, $N = 6$).

Vanadate 48 unidirectional fluxes and tissue uptake. Table 4 shows unidirectional fluxes and tissue uptake of vanadate 48 in the absence and in the presence of ouabain. The unidirectional flux of vanadate 48 from the serosal to mucosal solution was significantly greater than in the opposite direction, both in the presence and in the absence of ouabain. Tissue uptake was 30 times greater from the serosal solution than it was from the mucosal solution. In experiments in which ouabain was not added, we examined the effect of vanadate on sodium transport measured as SCC. Vanadate, 10^{-4} M, in the serosal solution caused a significant decrease in SCC as compared to controls (97.8 ± 6.1 vs. 52.8 ± 4.3%, $P < 0.001$). Baseline SCC was not different between control and experimental hemibladders (287 ± 44 and 263 ± 32 μA, NS). The decline in SCC by vanadate was accompanied by a proportional decline in PD from 35.7 ± 6.7 to 19.4 ± 4.3 mV, $P < 0.001$, leaving the resistance unchanged (117.2 ± 16.1 vs. 120.6 ± 15.3 ohm × 8 cm^2, NS). In control hemibladders, PD and resistance remained unchanged (35.7 ± 6.4 and 34.2 ± 65 mV and 125.5 ± 15.5 and 127.7 ± 14.7 ohm × 8 cm^2, NS). Addition of vanadate, 10^{-4} M, to the mucosal solution failed to alter SCC (97.8 ± 6.1 vs. 91.3 ± 6.1%, NS).

Vanadate uptake by isolated epithelial turtle bladder cells. Table 5 shows the uptake of vanadate 48 by isolated epithelial turtle bladder cells at various times of incubation with the isotope. Vanadate uptake tended to increase with time but the values at 20 min were not significantly different from those seen at 5 min.

Effect of vanadate on calcium uptake and calcium efflux. Because vanadate has been shown to inhibit calcium ATPase as well as other mechanisms involved in cell calcium regulation [9, 28], it was theoretically possible that vanadate might alter intracellular calcium either by promoting calcium uptake or inhibiting calcium efflux. Calcium 45 uptake was not significantly different between control and vanadate-treated hemibladders (control, 20.3 ± 2.25; 10^{-4} M vanadate 23.30 ± 1.78 nmoles/mg protein/5 min; $N = 8$; NS). Calcium efflux was also not affected by vanadate: at 5 min the rate coefficient of calcium efflux was not significantly different between control and vanadate-treated hemibladders (control, 1.38 ± 0.11; vanadate, 1.28 ± 0.23%; $N = 6$; NS). At all subsequent times, the rate of calcium efflux was also not different between the control and vanadate-treated bladders.

Discussion

In recent years, the effect of vanadate on several transport processes has been studied extensively [1–9]. This compound inhibits Na-K-ATPase by blocking a conformational change that must follow the hydrolysis of the phosphoenzyme. Vanadate has also been shown to inhibit the proton ATPase of fungi. We sought, therefore, to characterize the effect of vanadate on urinary acidification by the turtle bladder [14–16]. Urinary acidification in the turtle bladder is influenced by electrochemical gradients, availability of substrate and carbon dioxide and

Table 3. Effect of vanadate (10^{-4}) on lactate production and ATP levels in the presence of mitochondrial inhibition ($N = 7$)[a]

Group	H^+ secretion, μA			Lactate production, $\mu moles/hr$			ATP levels *nmoles/mg of protein*
	B	*P*	E	B	*P*	E	
Control	17.7 ± 5.1	NS	19.6 ± 4.8	1.69 ± 0.37	NS	1.72 ± 0.16	2.56 ± 0.44 ($N = 6$)
	NS		NS	NS		< 0.025	NS
Vanadate, 10^{-4}	20.1 ± 6.3	< 0.02	13.4 ± 5.2	1.31 ± 0.28	< 0.025	2.27 ± 0.15	2.74 ± 0.58 ($N = 6$)

[a]Values are the means ± SEM. B is baseline; E, values obtained 90 min after addition of the vehicle or vanadate.

Table 4. Vanadate 48 unidirectional fluxes and tissue uptake in absence and presence of ouabain[a]

Ouabain	Unidirectional flux, *pmoles/60 min/mg tissue wt*			Net flux	Tissue uptake		
	Serosa→mucosa		Mucosa→serosa		Serosa		Mucosa
−	16.98 ± 0.99	$P < 0.05$[b]	8.83 ± 5.05	7.29 ± 3.5 ($N = 8$)	117.58 ± 3.53 ($N = 9$)	$P < 0.01$	3.61 ± 1.08 ($N = 9$)
+	27.89 ± 9.00	$P < 0.005$	16.236 ± 8.42	11.84 ± 1.76 ($N = 7$)	106.05 ± 4.72 ($N = 8$)	$P < 0.001$	2.87 ± 0.548 ($N = 8$)

[a] Values are the means ± SEM.
[b] *P* value is calculated by the one-tail *t* test.

Table 5. Vanadate 48 uptake by isolated turtle bladder epithelial cells[a]

Group	$^{48}VO_4$ uptake, *pmoles/mg of protein*		
	5 min	10 min	20 min
Vanadate, 10^{-4}	0.427 ± 0.172 ($N = 5$)	0.548 ± 0.134 ($N = 5$)	0.621 ± 0.139 ($N = 6$)
Vanadate, 10^{-5}	0.130 ± 0.041 ($N = 6$)	0.248 ± 0.021 ($N = 6$)	0.249 ± 0.087 ($N = 6$)

[a] Values are the means ± SEM.

certain hormones [14–16]. In the present experiments, these variables were carefully controlled, and thus the observed changes cannot be accounted for by alterations in these parameters.

Vanadate caused a significant inhibition of hydrogen ion secretion and of sodium transport. The inhibitory effect of vanadate on sodium transport in the turtle bladder was similar to that observed in the toad bladder [29]. Vanadate has been shown recently to inhibit the bicarbonate moiety of the anion current in the turtle bladder and hydrogen ion secretion by the toad bladder [30–32]. The relative magnitude of the inhibitory effect of vanadate on hydrogen ion secretion was the same in the presence and in the absence of mitochondrial function; thus, suggesting that this effect of vanadate cannot be solely accounted for by inhibition of oxidative phosphorylation. These observations suggest that vanadate does not inhibit hydrogen ion secretion by blocking mitochondrial ATPase in the turtle bladder. This contention is in agreement with the recent demonstration that vanadate fails to inhibit mitochondrial ATPase in other tissues [9]. It cannot be excluded, however, that under aerobic conditions vanadate inhibits hydrogen ion secretion both by impairing oxidative phosphorylation and by inhibiting the pump itself or some other metabolic step essential for hydrogen ion secretion. In agreement with the possibility that the vanadate may interfere with oxidative phosphorylation is the finding that vanadate decreased ATP levels under aerobic conditions. Because hydrogen ion pump is likely a proton ATPase, inhibition of hydrogen ion secretion by a direct effect on the pump should decrease the utilization of ATP and result in a rise in ATP levels and in the ATP/ADP ratio. In the present study, no change in the ATP/ADP ratio was observed, suggesting that vanadate may have more than one effect; that is, it may decrease ATP utilization by the pump but it may simultaneously decrease ATP production leaving the total amount of ATP unchanged. Furthermore, it is possible that vanadate may not be a specific inhibitor of hydrogen ion pump and may have a large number of sites of action, such as the pentose phosphate shunt or other metabolic pathways.

The effect of vanadate on hydrogen ion secretion was only disclosed when vanadate was added to the serosal side. The lack of an inhibitory effect of vanadate with mucosal addition can be explained by the fact that vanadate uptake was 30 times greater from the serosal side than it was from the mucosal side. These observations indicate that vanadate is taken up from the serosa and likely must enter the cell to exert its inhibitory effect on hydrogen ion secretion.

The unidirectional fluxes of vanadate are indicative of a net serosa to mucosa flux, suggesting secretion of vanadate into the lumen. That vanadate crosses the luminal membrane when added to the serosa but failed to do so when added to the mucosa suggests the existence of active secretion of vanadate in the turtle bladder. Further studies are necessary to evaluate this possibility. The unidirectional fluxes of vanadate tended to be higher in the presence of ouabain than in control. The reason for this finding is unclear.

Inasmuch as vanadate has been shown to alter calcium efflux in other systems [29], and because an increase in cytosolic calcium [18–20] is capable of inhibiting hydrogen ion secretion, we examined the effect of vanadate on calcium uptake and efflux. Vanadate failed to alter these variables, suggesting that alterations in cytosolic calcium likely do not account for the

effect of vanadate on hydrogen ion secretion. That vanadate decreased hydrogen ion secretion in presence of inhibition of carbonic anhydrase and microtubule function suggests, but does not prove, that vanadate inhibits hydrogen ion secretion by a mechanism other than inhibition of carbonic anhydrase or microtubule function [21, 27].

To gain insight into the mechanism of the inhibitory action of vanadate on hydrogen ion secretion, we examined the effect of this compound on the proton motive force, an estimate of the force of the hydrogen ion pump [22]. Vanadate significantly decreased this parameter. In this context, it is interesting to note that addition of 5% carbon dioxide to the serosal solution failed completely to stimulate hydrogen ion secretion in vanadate-treated bladders. Addition of 5% carbon dioxide to the serosal solution stimulates, at least temporarily, hydrogen ion secretion in bladders treated with carbonic anhydrase inhibitors, disulfonic stilbenes, or with metabolic inhibitors [21, 33, 34]. The failure of 5% carbon dioxide to stimulate hydrogen ion secretion in vanadate-treated hemibladders is in agreement with the suggestion that the proton pump may be impaired by vanadate. It should be emphasized, however, that a decrease in proton motive force need not necessarily indicate an effect on the pump as indicated by the fact that SITS alters this parameter without affecting the pump [34].

In the presence of mitochondrial inhibition and carbon dioxide, hydrogen ion secretion is influenced by the rate of glycolysis, the integrity of the pump, and the utilization of energy by the pump [14, 16]. The hydrogen ion pump is thought to be a proton translocating ATPase [15, 16], and thus the influence of glycolysis on hydrogen ion secretion is mediated by the availability of ATP. Under anaerobic conditions, the rate of lactate production can be inferred to reflect the rate of ATP synthesis by the glycolytic pathway. Sodium and hydrogen ion transport by the turtle bladder under anaerobic conditions have been shown to correlate closely with the rate of lactate production [25, 35]. The inhibition of hydrogen ion secretion by vanadate could result from several different mechanisms: *first,* vanadate could inhibit ATP synthesis by the glycolytic pathway; *second,* it could interfere with ATP utilization by the pump; *third,* it could inhibit the pump directly or secondarily through a metabolic effect.

Vanadate has been shown to increase the rate of glycolysis in several tissues [35–39]. An increase in the rate of glycolysis would be expected to increase the rate of lactate production, the rate of ATP synthesis, and the ATP/ADP ratio [40]. In the turtle bladder, vanadate also caused a significant increase in lactate production, but without the expected change in ATP/ADP ratio. These findings suggest that in the turtle bladder, like in other tissues, vanadate partially uncouples glycolysis from ATP synthesis [39]. This effect of vanadate, however, likely cannot account for the inhibition of hydrogen ion secretion since ATP levels were not significantly different from controls. Thus, it it likely that ATP availability was not rate limiting for hydrogen ion secretion. It is unclear why previous studies failed to demonstrate a decrease in ATP levels in turtle bladders under anaerobic conditions as compared to aerobiosis [25].

These observations suggest that the effect of vanadate on hydrogen ion secretion occurs at the level of the pump, either as the result of inhibition of hydrogen ion pump directly or from inadequate ATP utilization by the pump. Experimental distinction between these two possibilities may be currently impossible in the turtle bladder. Steinmetz, Husted, and Mueller [35] have reached a similar conclusion regarding the inhibitory effect of vanadate on hydrogen ion secretion by the turtle bladder.

In conclusion, our results suggest that vanadate has a number of effects that are capable of inhibiting acidification by the turtle bladder. Among these effects the inhibition of urinary acidification could be the result of inhibition of the pump, interference with utilization of ATP by the pump, or other metabolic effects.

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References

1. Beauge LA, Glynn IM: Commercial ATP containing traces of vanadate alters the response of (Na^+ + K^+) ATPase to external potassium. *Nature* 272:551–552, 1978
2. Cantley LC Jr, Resh MD, Guidotti G: Vanadate inhibits the red cell (Na^+, K^+) ATPase from the cytoplasmic side. *Nature* 272:552–554, 1978
3. Bowman BJ, Mainzer SE, Allen KE, Slayman CW: Effects of inhibitors on the plasma membrane and mitochondrial adenosine triphosphatase of Neurospora crassa. *Biochim Biophys Acta* 512:13–28, 1978
4. Cantley LC Jr, Cantley LG, Josephson L: A characterization of vanadate interactions with the (Na,K)-ATPase: Mechanistic and regulatory implications. *J Biol Chem* 253:7361–7368, 1978
5. Balfour WE, Grantham JJ, Glynn IM: Vanadate-stimulated natriuresis. *Nature* 275:768, 1978
6. Grantham JJ: The renal sodium pump and vanadate. *Am J Physiol* 239: F97-F106, 1980
7. Beauge L, Dipolo R: Vanadate selectively inhibits the K^+-activated Na^+ efflux in squid axons. *Biochim Biophys Acta* 551:220–223, 1979
8. Cantley LC Jr, Aisen P: The fate of cytoplasmic vanadium. Implications on (Na,K)-ATPase inhibition. *J Biol Chem* 254:1781–1784, 1979
9. O'Neal S, Rhoads DB, Racker E: Vanadate inhibition of sarcophasmic reticulum ATPase and other ATPases. *Biochem Biophys Res Comm* 89:845–850, 1979
10. Day H, Middendorf D, Lukert B, Heinz A, Grantham J: The renal response to intravenous vanadate in rats. *J Lab Clin Med* 96:382–395, 1980
11. Bowman BJ, Slayman CW: The effects of vanadate on the plasma membrane ATPase of neurospora crassa. *J Biol Chem* 254:2928–2934, 1979
12. Willsky GR: Characterization of the plasma membrane Mg^{2+}-ATPase from the yeast, saccharomyces cerevisiae. *J Biol Chem* 254:3326–3332, 1979
13. DuFour JP, Boutry M, Goffeau A: Plasma membrane ATPase of the yeast. *J Biol Chem* 255:5735–5741, 1980
14. Steinmetz PR: Cellular mechanisms of urinary acidification. *Physiol Rev* 54:890–956, 1974
15. Dixon T, Al-Awqati Q: Urinary acidification in the turtle bladder is due to a reversible proton translocating ATPase. *Proc Natl Acad Sci* 76:3135–3138, 1979
16. Steinmetz PR, Mueller A, Beauven R: Effect of ATPase inhibitors on anaerobic H^+ transport by turtle urinary bladder (abst). *Fed Proc* 38:1121, 1979
17. Arruda JAL, Dytko G, Mola R, Kurtzman NA: The mechanism of lithium induced renal tubular acidosis. *Kidney Int* 17:196–204, 1980
18. Arruda JAL: Calcium inhibits urinary acidification: Effect of

ionophore A23187 in the turtle bladder. *Pfluegers Arch* 381:107–111, 1979
19. ARRUDA JAL, SABATINI S: Cholinergic inhibition of urinary acidification by the turtle bladder. *Kidney Int* 17:622–630, 1980
20. ARRUDA JAL, SABATINI S: Effect of quinidine on Na, H and water transport by the turtle and toad bladders. *J Mem Biol* 55:141–147, 1980
21. SCHWARTZ JH: H^+ current response to CO_2 and carbonic anhydrase inhibition in turtle bladder. *Am J Physiol* 231:565–572, 1976
22. AL-AWQATI Q, MUELLER A, STEINMETZ PR: Transport of H^+ against electrochemical gradients in turtle urinary bladder. *Am J Physiol* 233:F502–508, 1977
23. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ: Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–275, 1951
24. KIMMICH GA, RANDLES J, BRAND JS: Assay of picomole amounts of ATP, ADP, and AMP using the luciferase enzyme system. *Anal Biochem* 69:187–206, 1975
25. KLAHR S, BRICKER NS: Energetics of anaerobic sodium transport by fresh water turtle bladder. *J Gen Physiol* 48:571–580, 1965
26. SIMONS TGB: Vanadate a new tool for biologists. *Nature* 281:337–338, 1979
27. ARRUDA JAL, SABATINI S, MOLA R, DYTKO G: Inhibition of H^+ secretion by colchicine and vinblastine. *J Lab Clin Med* 96:450–459, 1980
28. DIPOLO R, ROJAS HR, BEAUGE L: Vanadate inhibits uncoupled Ca efflux but not Na-Ca exchange in squid axons. *Nature* 281:229–230, 1979
29. DESOUSA RO, GROSSO A: Vanadate blocks cyclic AMP-induced stimulation of sodium and water transport in amphibian epithelia. *Nature* 279:803–804, 1979
30. EHRENSPECK G: Vanadate induced inhibition of sodium transport and of sodium independent anion transport in turtle bladder. *Biochem Biophys Acta* 601:427–429, 1980
31. FANESTIL DD: Vanadate nonselective inhibition of transepithelial Na^+, H^+ and water transport. *Experientia* 36:1045–1046, 1980
32. BEAUWENS R, CRABBÉ J, RENTMEESTERS M: Effect of vanadate on the functional properties of the isolated toad bladder. *J Physiol (Lond)* 310:293–305, 1981
33. SCHWARTZ JH, STEINMETZ PR: Metabolic energy and pCO_2 as determinants of H^+ secretion by turtle urinary bladder. *Am J Physiol* 233:F145–F149, 1977
34. COHEN L, MUELLER A, STEINMETZ PR: Inhibition of the bicarbonate exit step in urinary acidification by a disulfonic stilbene. *J Clin Invest* 61:981–986, 1978
35. STEINMETZ PR, HUSTED RF, MUELLER A: Relationship between H^+ transport and anaerobic glycolysis in turtle bladder: Vanadate sensitivity of H^+ pump (abst). *Clin Res* 28:560, 1980
36. TOLMAN E, BARRIS E, BURNS M, PANSINI A, PARTRIDGE R: Effect of vanadium on glucose metabolism in vitro. *Life Sci* 25:1159–1164, 1979
37. SHECHTER Y, KARLISH SJ: Insulin-like stimulation of glucose oxidation in rat adipocytes by vanadyl (IV) ions. *Nature* 284:556–558, 1980
38. DUBYAK GR, KLEINZELLER A: Insulin-mimetic effects of vanadate on rat adipocytes. *J Biol Chem* 255:5306–5312, 1980
39. DEMASTER EG, MITCHELL RA: A comparison of arsenate and vanadate as inhibitors or uncouplers of mitochondrial and glycolytic energy metabolism. *Biochemistry* 12:3616–3621, 1973
40. DURBIN R, MICHELANGELI F, NICKEL A: Active transport and ATP in frog gastric mucosa. *Biochim Biophys Acta* 367:177–189, 1974